Changes in Cytokine Profile During Local IL-2 Therapy in Cancer Patients

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Abstract. *Background: Exogenous interleukin 2 (IL-2) can influence the complex cytokine network in vivo. This study investigated the cytokine profile of patients with different malignancies before and after local IL-2 administration. Patients and Methods: The human TH1 / TH2 cytometric bead array (CBA) kit was used to investigate IL-2, IFNγ, TNFα, IL-4, IL-5 and IL-10 in a control group and in 13 patients. Results: The baseline serum IL-4 levels in patients were lower than in healthy controls, while the baseline ascitic IL-10 levels in patients was higher than in serum. The IL-2 applications induced a strong serum increase in IL-2 and IL-5 and even more in ascites, while IL-10 increased weakly and mainly locally. One month after the start of therapy, the serum IFNγ had increased in patients, reaching the level of the control group. Conclusion: After local injection, IL-2 probably leaks into the blood circulation. The higher increases of IL-2, IL-5 and IL-10 in ascites compared to the serum suggests that the injected cytokines and their effects are mainly local. The minor increase of the immunosuppressive IL-10 could explain the therapeutic difference between local and systemic IL-2 therapy since IL-10 levels markedly increase after systemic IL-2 therapy. IL-5 was always increased after IL-2 therapy and, consequently, may be a downstream mediator of antitumour responses.*

Local administration of interleukin 2 (IL-2) led to good therapeutic results in animal experiments (1-3) and in veterinary cancer subjects (3, 4). Therapeutic effects were also observed in patients with metastasized lung cancer, ovarian cancer, breast cancer, gastrointestinal malignancies (5-8), hepatocellular carcinoma (HCC) (9), mesothelioma (10), recurrent bladder carcinoma (11) metastasized nasopharyngeal carcinoma (12) and pulmonary metastatic renal-cell carcinoma (13).

Systemic IL-2 application is, however, associated with many adverse effects and marked toxicity. A well-known effect of IL-2 is vascular leakage, which severely limits its systemic application. However, local administration of IL-2 results in higher response rates than systemic IL-2 regimes and causes few adverse effects (5-7, 12). Local application has apparent advantages such as higher concentrations of IL-2 at the tumour site and reduce systemic adverse effects due to lower serum concentrations.

Establishment of the immunological changes related to administered IL-2 might provide information on the antitumour mechanism of this cytokine *in vivo*. One of the interesting aspects of this immunological effect is the influence of IL-2 on the complex cytokine network. Various cytokines exert cross-regulatory immunological effects and are produced by different cells *in vivo*. Some of them, classified as Th1-like cytokines (IL-2, IL-12, IFNγ), play important roles in cell-mediated immune reactions. Others, classified as Th2-like (IL-4, IL-5, IL-13, and probably IL-9), are involved in humoral immune responses (14). This cytokine nomenclature emphasizes the function of the cytokines rather than their cell source. There are also cytokines that do not exactly fit into these two categories. IL-10 is an anti-inflammatory cytokine with Th1-immuno-supressive effects (15, 16), but it also down-regulates Th2 responses (17). TNFα is a pro-inflammatory cytokine which is often produced in the absence of specific immunity and can have different effects on the Th1 – Th2-like cytokine balance (18).

In different diseases, the relative predominance, but not an absolute presence or absence, of Th1-like or Th2-like cytokines has been documented. A predominance of Th2-like cytokines was registered in several tumour types – basal and squamous cell carcinomas, Sezary syndrome, some lymphomas, multiple myeloma, Kaposi's sarcoma, gliomas, melanoma and gastrointestinal malignancies (14, 19-23).

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Table I. *Patients' tumour status.*

Patients	13		
Male	6		
Female	7		
Age (mean age)	47–81 years (60)		
Tumor type		Stage	Number of patients with malignant ascites
Gastric cancer	2	IV, 0*	-
HCC	2	III, IV	-
Liver angiosarcoma	1	IV	1
Abdominal mesothelioma	1	IV	-
Breast cancer	2	IV	1
Ovarian cancer	1	IV	-
Colon cancer	4	IV	1
Previous therapy			
Resection of the primary tumour	7		
Standard chemotherapy***	5		
Hormonotherapy***	4		
PEI or HFTT**	5		

*0: the patient was radically operated on for gastric cancer.
**HFTT (high frequency thermotherapy) and PEI (percutaneous ethanol injection) had been conducted 10 days before the first IL-2 application in order to induce necrosis.
***Standard chemotherapy and hormonotherapy had been conducted more than 3 months before the start of IL-2 therapy.

Table II. *IL-2 therapy – schemes, routes and doses of application.*

Scheme of application	No. of patients
Once weekly	8
Once monthly	5
Total dose per application (range 1.5 – 18 MIU)	
1.5 MIU	1
4.5 MIU	8
4.5 MIU; 9 MIU*	2
9 MIU	1
9 MIU; 18 MIU*	1
Route of application	
Intraperitoneally (*i.p.*)	5
i.p. into malignant ascites	3
i.p. into artificially made ascites	2
Intratumorally (*i.tu.*)	5
Into primary sigmoidal tumour	2
Into primary or metastatic liver tumour	2
Into peritoneal metastases	1
i.p. into artificially made ascites + *i.tu.* into peritoneal metastases	1
Intra-arterially	2
Into the hepatic artery	2

*Three patients received two different dose-levels during the first and second applications.

There are also data suggesting the involvement of cell-mediated immune responses in rejecting different tumours (7, 24). The antitumour effect of local IL-2 in clinical trials is not completely understood, but its *in loco* administration can influence different cells and lead to the production of various cytokines involved in cell-mediated or humoral immune responses. INFg, TNFα, IL-2 and IL-12 can be secreted by Th1 lymphocytes, type I CD8+ lymphocytes, NK cells, monocytes / macrophages, dendritic cells, B cells and neutrophils. Other cytokines (IL-4, IL-5, IL-6, IL-13 and probably IL-9) can be secreted by Th2 lymphocytes, type II CD8+ lymphocytes, monocytes / macrophages, B cells, mast cells, eosinophils and some tumour cells (14).

The aim of the present study was to investigate the cytokine profile of patients with different malignancies at the baseline, as well as to register possible cytokine changes in the serum and ascites after local IL-2 administration in order to establish specific immunological changes.

Patients and Methods

Patients. Thirteen patients with primary or metastatic gastrointestinal cancer were enrolled in the study involving the local application of IL-2 (Proleukin, Chiron, Amsterdam, The Netherlands), undertaken at the University Gastroenterology Department in Sofia, Bulgaria. The protocol was approved by the local Ethical Committee and all patients signed written consent. In all cases there was a histopathological diagnosis. Patients entered the study if they had normal renal function and lack of infection, severe cardiac, endocrine or pulmonary disease. Most patients who were admitted to this IL-2 trial were advanced cases with progressive disease (stage III-IV), either inoperable or not fit for standard chemo- or radiotherapy at the time of admission. One patient was radically operated on for gastric cancer (the primary tumour and the metastatic locoregional lymph nodes were removed), although he did not have any other metastases. The patient refused the proposed chemotherapy but agreed to local IL-2 therapy. A clinical oncologist was always consulted to exclude that further radio- or chemotherapy was still a valid option. The patients are characterised in Table I.

Our primary intention was to apply IL-2 as close as possible to the tumour (primary or metastatic) and at the site of the antitumour immune reaction. IL-2 was injected directly into the primary tumour or metastases, under ultrasound and endoscopic control whenever possible, into the hepatic artery under X-ray control, or intraperitoneally (*i.p.*) under ultrasound control (directly into malignant ascites or into artificial ascites). Artificial ascites was created by the infusion of approximately 2000 ml of 0.9% saline solution into the peritoneal cavity through a fine needle under ultrasound control. IL-2 was administered either once

weekly or once monthly in doses between 1.5 and 18 MIU. The dose-levels and schedules are presented in Table II. In five patients IL-2 applications were stopped due to patients' refusal to receive further treatment because of non-medical reasons (two patients), severe deterioration of the disease (two patients) or patient death (one patient). None of the patients stopped IL-2 therapy because of inconvenience or side-effects related to the therapy.

Drug toxicity and adverse effects during IL-2 therapy were evaluated by routine haematological analysis, liver function tests (ASAT, ALAT, GGT, ALP, bilirubin), creatinine, blood glucose and clinical observation of the patients.

CBA cytokine testing. The human TH1/ TH2 Cytometric Bead Array (CBA) Kit (BD Biosciences Pharmingen, San Diego, USA) was used to investigate IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα in a control group of 36 healthy subjects and in 13 patients before and after IL-2 applications. The BD CBA measures soluble analytes in a particle-based immunoassay and uses fluorescence detection by flow cytometry. The sensitivity is comparable to conventional ELISA. The detection limits were 2.6 pg/ml for IL-2, 2.6 pg/ml for IL-4, 2.4 pg/ml for IL-5, 2.8 pg/ml for IL-10, 2.8 pg/ml for TNFα and 7.1 pg/ml for IFNγ. All procedures were performed according to the kit manual. The cytokines were quantitatively measured in the serum and, when possible, in ascites.

Serum cytokine levels were measured in a control group of 36 healthy subjects who included 16 males and 20 females matched by age with that of the patients (mean age 59 years, range 43-88 years). Ten patients were matched with three controls of the same age; the other three patients were matched with only two controls.

In all 13 patients, serum IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα were measured at baseline (0 h) and 24 h after the first IL-2 application. Additionally 1 month after initiation of IL-2, serum cytokine levels were measured in eight patients before and 24 h after the second IL-2 application. After centrifugation, all serum samples were stored at –70˚C until the assay.

Ascitic samples could be taken only from three patients with once weekly *i.p.* applications into malignant ascites prior (0 h) and 24 h after the first IL-2 application. In patients with artificially made ascites, ascitic samples could not be taken, due to the insufficient amount of fluid 24 h after the applications. The ascitic fluid was centrifuged to remove cells and was stored at –70˚C until the assay.

Statistical analysis. All data that were below the detection limits were set at half of the detection limit. Afterwards, for the statistical analysis, all the data were log-transformed for calculation of the averages, SDs and SEMs. The actual data were recalculated from the log-transformed averages. Statistical significance was set at the $p<0.05$ level and biological significance was set at double or half values from appropriate controls. The Student's *t*-test was used to compare the serum cytokine levels in patients with controls at all time-points of the investigation. The paired *t*-test was used to compare serum and ascitic cytokine levels at different time-points in the same patients and to compare serum and ascitic cytokine levels with each other at the same time-point in the same patient. The time-points compared were: before and 24 h after the first application, before and 24 h after the second application, before both applications and 24 h after both applications.

Results

Side-effects. No severe adverse effects were observed in any patient. Mild fever, nausea, abdominal pain and malaise were the most common complaints, which were resolved 24 to 48 h after application. No significant changes were registered for RBC, WBC, haemoglobin or haematocrit. An increase in eosinophils and decrease in lymphocytes 24 h after IL-2 applications were observed. No significant changes in the other examined laboratory parameters were registered.

IL-2. IL-2 was detectable in the serum (above 2.6 pg/ml) of 14 out of 36 healthy controls, but it was below the detection limit before each IL-2 application in all 13 patients. At these time-points, the serum IL-2 levels in patients were lower than in the controls ($p=0.0001$). Twenty-four hours after IL-2 administration, the serum IL-2 levels in patients were 3.5 times higher than in the controls ($p<0.05$).

With regard to the patient data, 24 h after IL-2 administration this cytokine had increased approximately 5-fold in the serum compared to the levels before both administrations ($p\leq0.02$, Figure 1).

Different IL-2 application routes resulted in different serum IL-2 concentrations per 1 MIU infused after 24 h. Administration of IL-2 into the peritoneal cavity, the tumour and the hepatic artery resulted in 2.7, 0.9 and 0.2 pg IL-2 per ml per 1 MIU infused, respectively (all differences $p<0.05$).

IFNγ. IFNγ was detectable in the serum (above 7.1 pg/ml) in 14 out of 36 healthy controls, but only in two out of 13 patients before therapy. The serum baseline levels in patients were 40% lower than in the control group ($p=0.008$). During the other time-points of investigation, the IFNγ levels did not statistically differ between the patients and controls.

In patients, no obvious or significant changes were found in the serum IFNγ levels before and 24 h after IL-2 administration. One month after initiation of therapy, the serum IFNγ had increased 1.7-fold in patients compared to the baseline values ($p=0.02$), thus being similar to those of the control group (Figure 2).

IL-4. IL-4 was detectable in the serum (above 2.6 pg/ml) in 28 out of 36 healthy controls, but only in three out of 13 and one out eight patients before the first and second applications, respectively. At all time-points of investigation, the serum levels of IL-4 in patients were approximately 60% lower than in the controls ($p<0.05$). At all time-points after IL-2 administration, IL-4 levels had not changed significantly in the serum (Figure 3).

IL-5. IL-5 was detectable in the serum (above 2.4 pg/ml) in twelve out of 36 healthy controls, but only in one out of 13 and one out of eight patients before the first and the second IL-2 administrations, respectively. At baseline, the serum

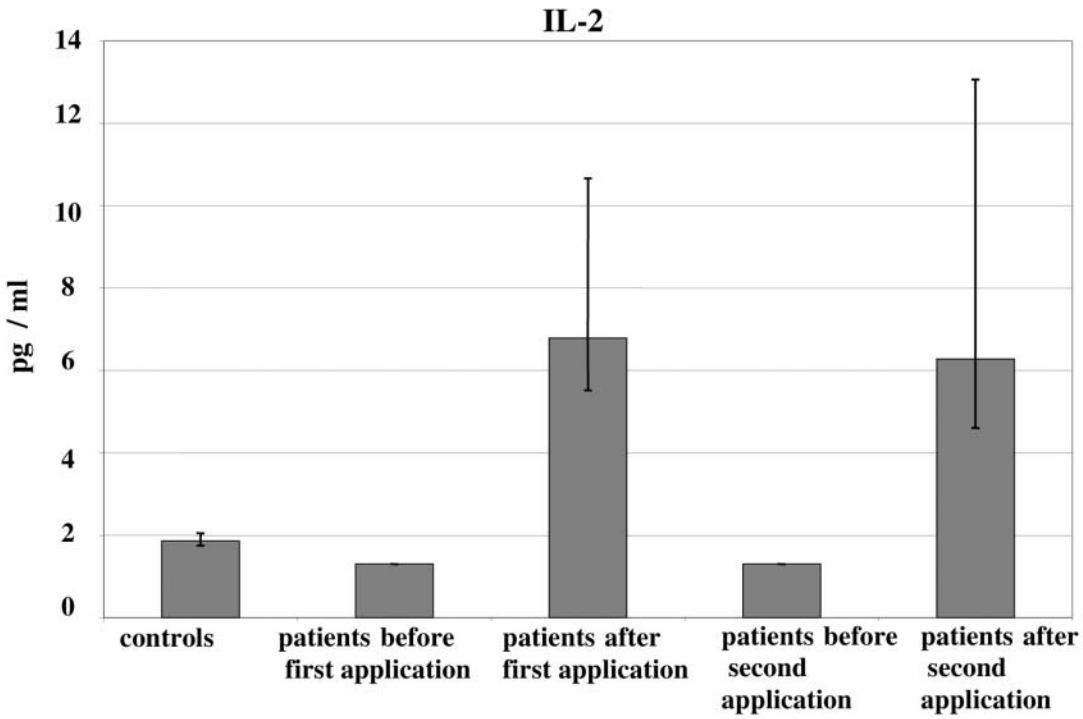

Figure 1. *Serum IL-2 concentrations in controls and patients at different time-points of investigation.*

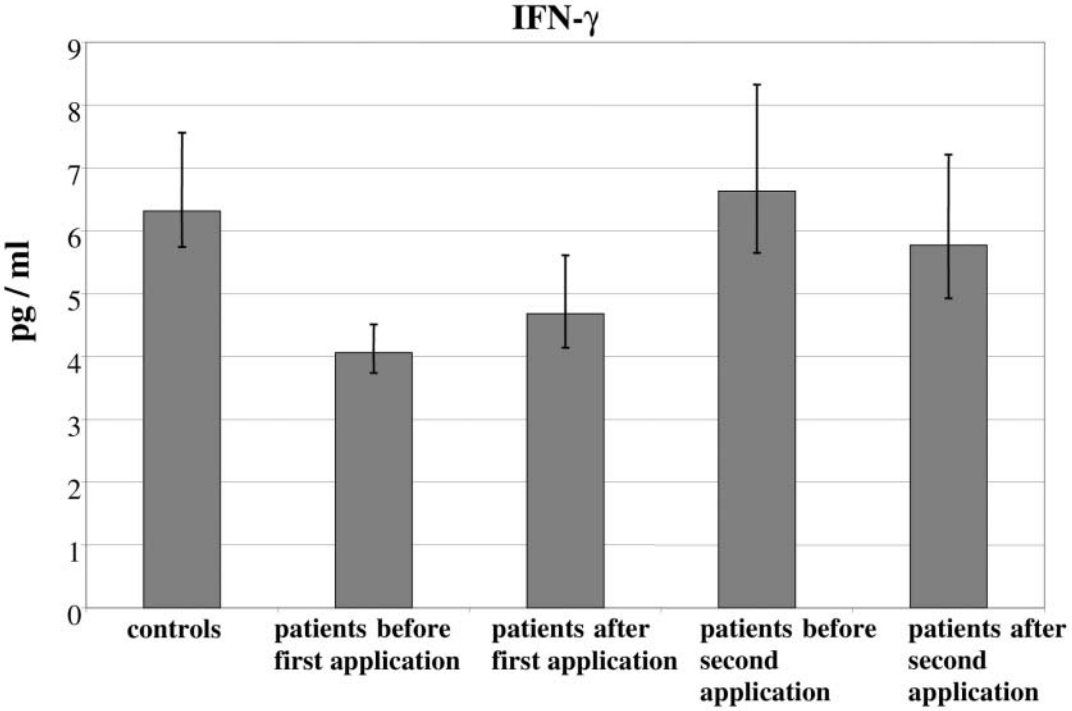

Figure 2. *Serum IFN-γ concentrations in controls and patients at different time-points of investigation.*

levels of IL-5 were 30% lower in patients compared to the controls, showing statistical significance ($p=0.010$). After the first and second IL-2 administrations, the serum IL-5 had increased 3.2- ($p=0.0002$) and 9-fold ($p=0.001$), respectively, and was 2.3- and 7.7-fold higher, respectively, in the patients than in the controls ($p=0.01$) (Figure 4).

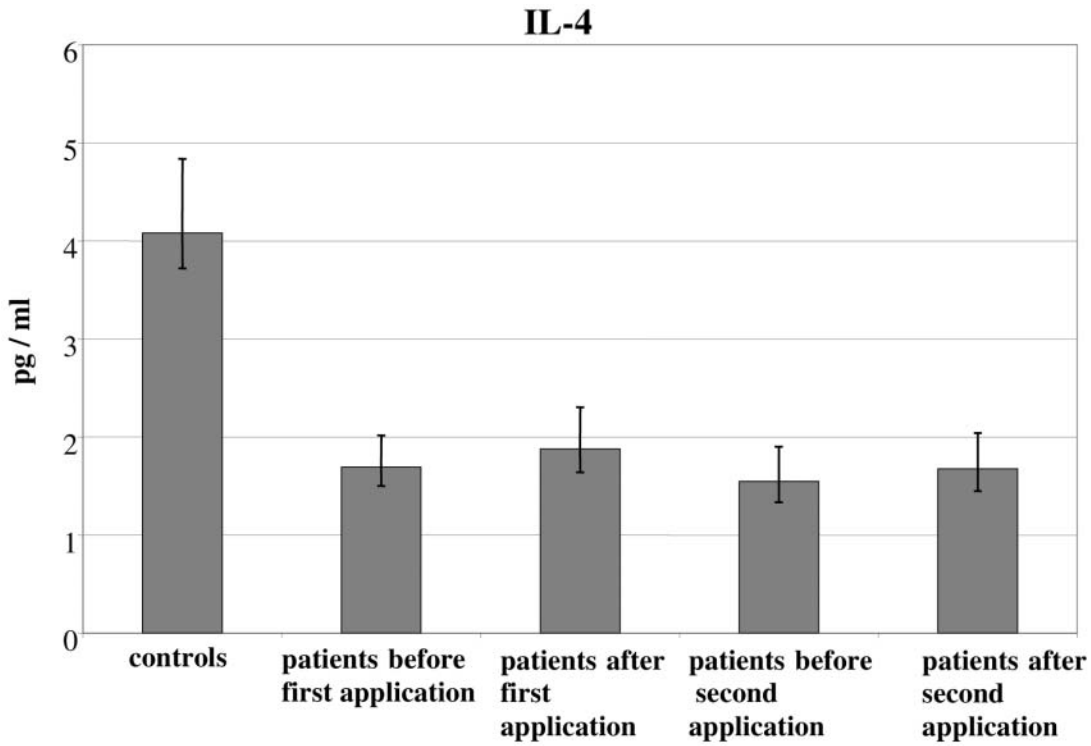

Figure 3. *Serum IL-4 concentrations in controls and patients at different time-points of investigation.*

IL-10. IL-10 was detectable in the serum (above 2.8 pg/ml) in 18 out of 36 healthy controls and in six out of 13 patients at the baseline and in six out of eight patients before the second IL-2 application. At these time-points, the serum levels in patients and the controls did not differ statistically. Twenty-four hours after the first and second IL-2 administrations, the serum IL-10 levels were 1.9- and 2.2-fold higher, respectively, in patients than in controls ($p=0.07$ and $p=0.02$) (Figure 5). The differences before and after IL-2 therapy were only 1.7-fold for both applications ($p=0.02$ and $p=0.09$, respectively).

TNFα. TNFα was detectable in the serum (above 2.8 pg/ml) in 15 out of 36 healthy controls, but only in one out of 13 and one out of eight patients before both IL-2 applications. At all time-points of the investigation, the serum levels of this cytokine were 30-35% lower in patients than in the controls ($p<0.05$). No significant changes in TNFα were registered in the serum either 24 h after IL-2 application or 1 month after the start of therapy compared to the baseline (Figure 6).

Ascites. At baseline, the ascitic levels of IL-2, IL-5, TNFα and IFNγ were below the detection limit in the three tested patients, while IL-4 was detectable only in one patient. In all three tested patients, the ascitic levels of IL-10 were detectable and were 12-fold higher than in the patients' sera ($p=0.03$). Thus, the baseline ascitic cytokine levels were similar to those found in serum, except for IL-10 which was higher in ascites (Figure 7).

Twenty-four hours after *i.p.* IL-2 application, a huge increase in IL-2 was detected (534-fold), while a large increase in IL-5 (44-fold) and a small increase in IL-10 (1.9-fold) were observed ($p=0.005$, $p=0.007$ and $p=0.002$, respectively). The other measured cytokines did not change significantly (Figure 8). Twenty-four hours after IL-2 application, the ascitic levels of IL-2, IL-5 and IL-10 were, respectively, 53-, 9.4- and 16-fold higher compared to the serum ($p=0.005$, $p=0.02$ and $p= 0.005$ respectively, Figure 9).

Discussion

Baseline results. Baseline serum levels of IL-2, IFNγ, TNFα, IL-4 and IL-5 in the patients were statistically significantly lower in comparison with the healthy controls. This difference was biologically significant only for IL-4. The baseline serum IL-10 concentrations were in the same range in patients and controls. The lower baseline levels of IL-2 in patients were statistically significant, but not according to the biological criteria, due to the low values in the controls. The fact that IL-2 was not detected in any patients' sera may indicate that it is indeed decreased in tumour patients. In our study, IFNγ and TNFα

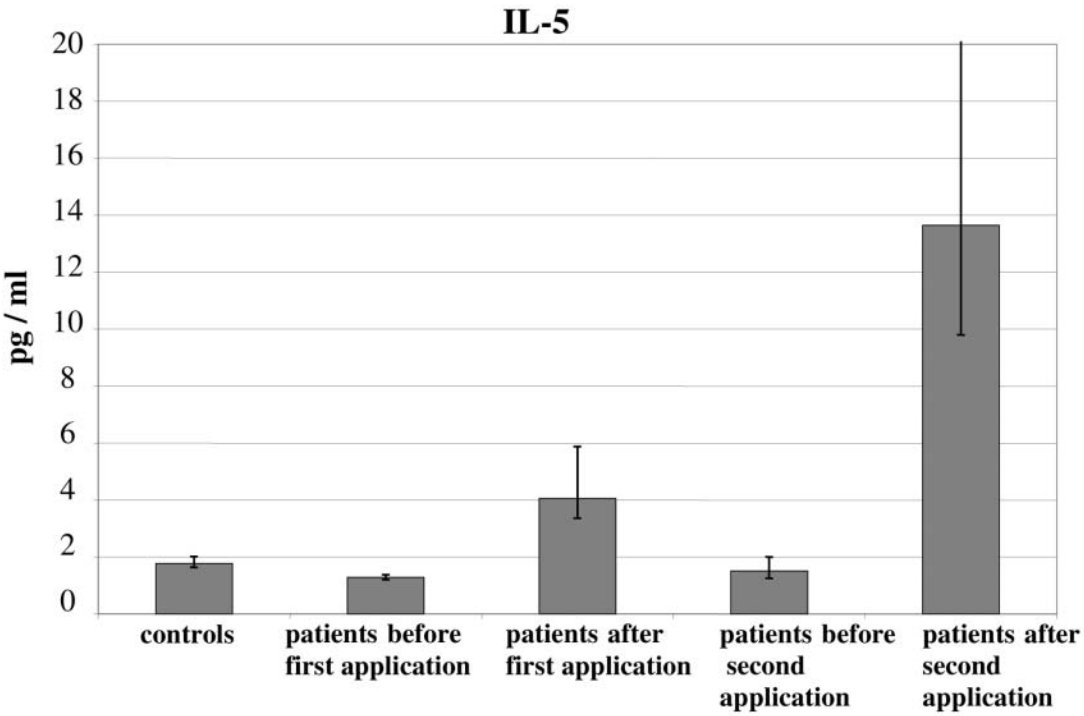

Figure 4. *Serum IL-5 concentrations in controls and patients at different time-points of investigation.*

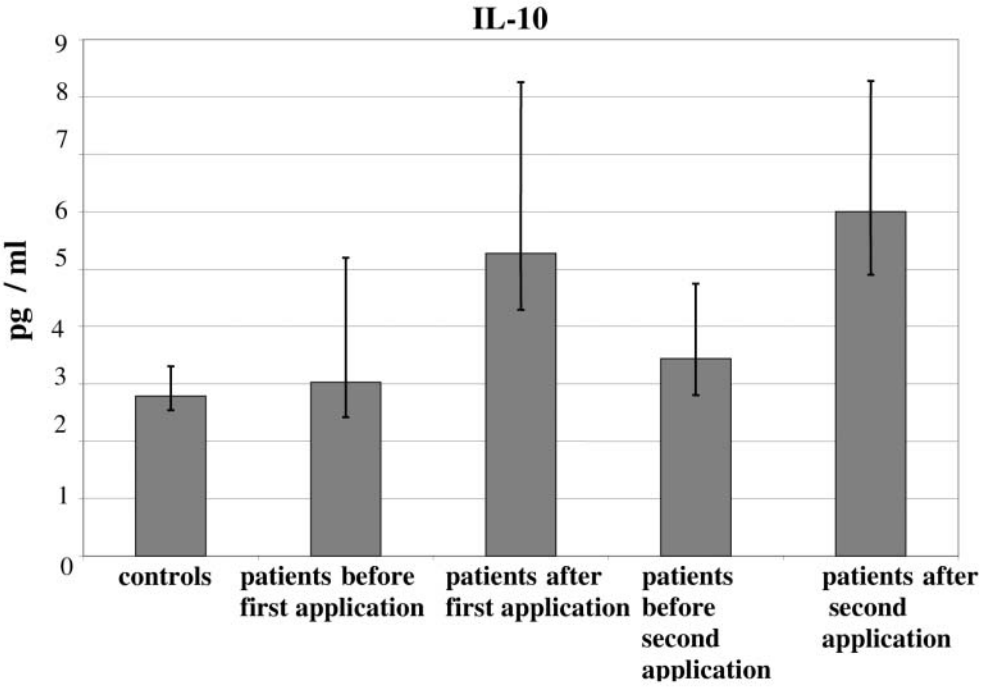

Figure 5. *Serum IL-10 concentrations in controls and patients at different time-points of investigation.*

did not allow any concrete biological conclusions to be drawn, but statistically they are decreased in patients prior to IL-2 therapy. The decrease in IL-5 in untreated patients was also statistically, but not biologically, significant. The data regarding IL-4 did allow biological or statistical conclusions to be drawn: this cytokine was reduced in the serum of tumour patients.

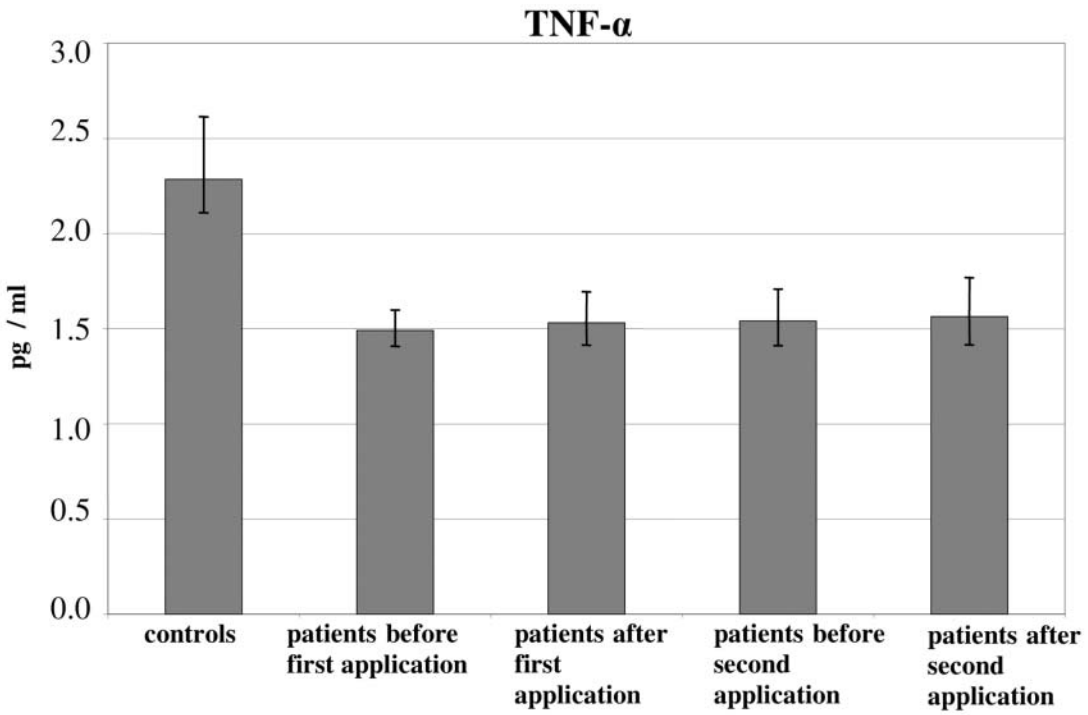

Figure 6. *Serum TNF-α concentrations in controls and patients at different time-points of investigation.*

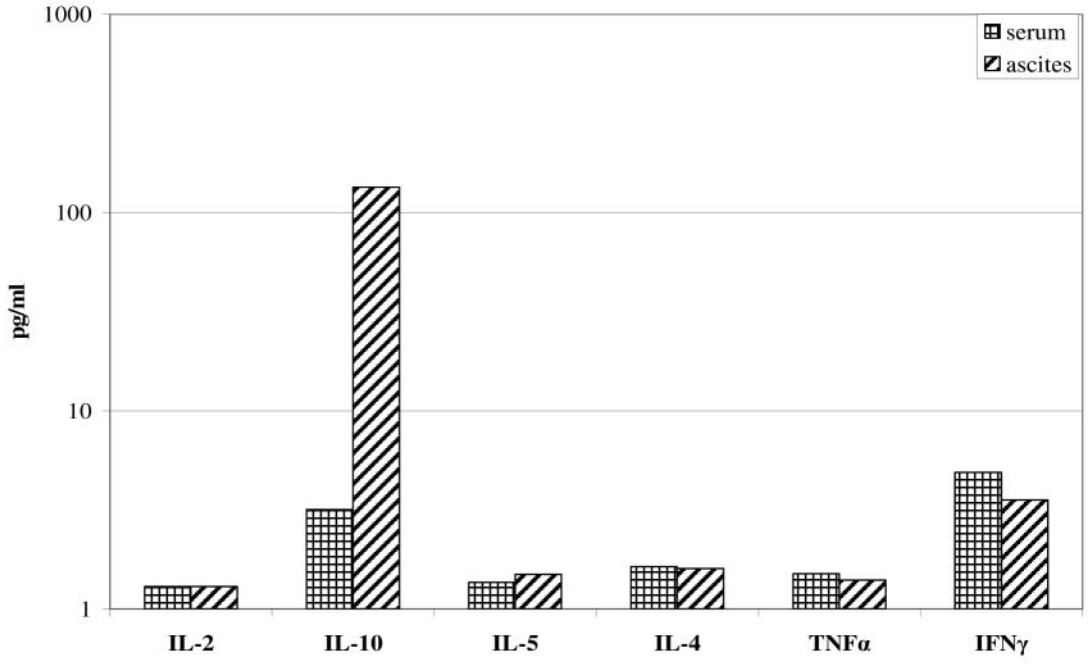

Figure 7. *Serum and ascitic cytokine levels at the baseline.*

Serum IL-10 concentrations were similar in patients and controls. In the malignant ascites of the three tested patients, a significantly higher level of IL-10 was found, while the other measured cytokines were in the same range as in the serum.

Other researchers have found decreases in IL-2 and IFNγ in patients with various tumours and the systemic or local predominance of type 2 (IL-4, IL-6) or regulatory (IL-10) cytokines (14, 19-25). Our results suggested that, in

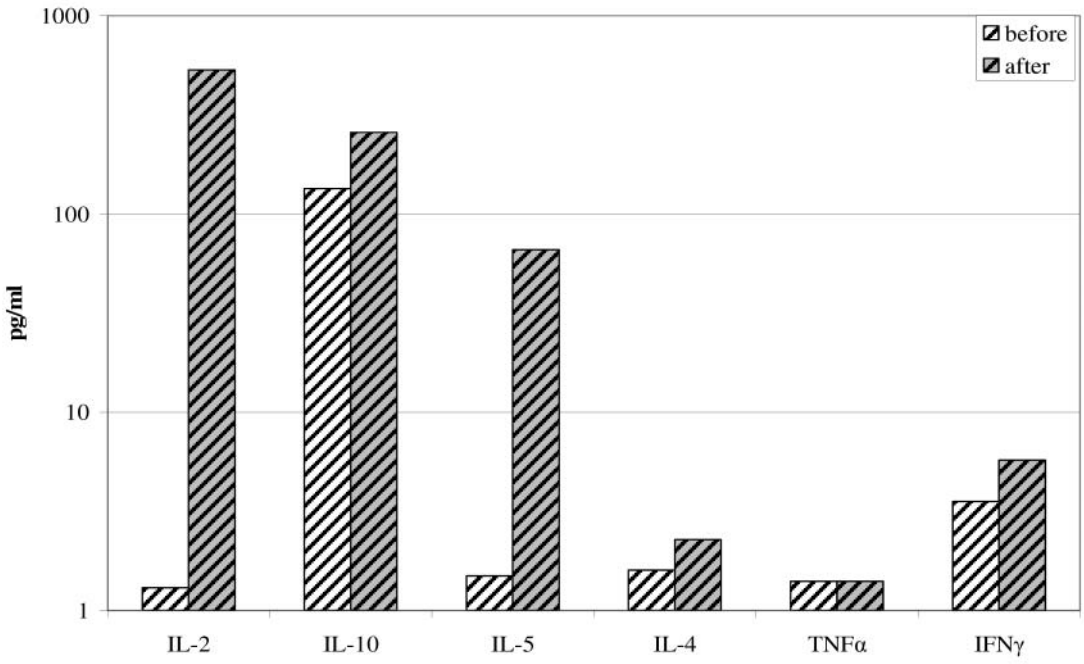

Figure 8. *Changes in ascitic cytokine levels before and 24 h after IL-2 application.*

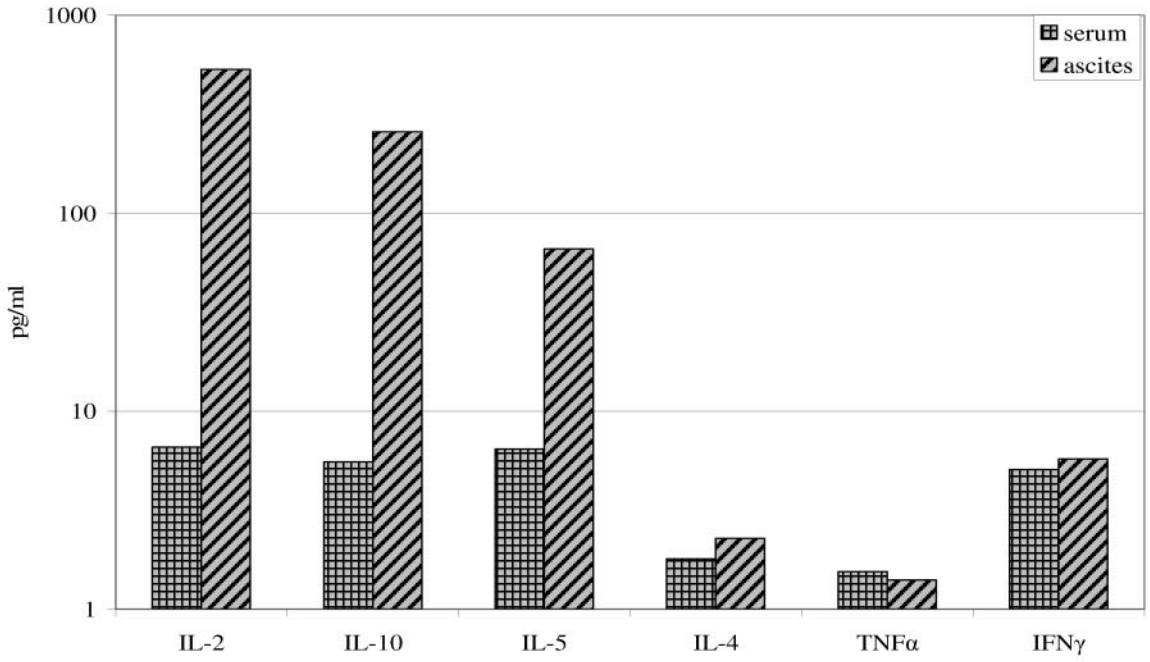

Figure 9. *Serum and ascitic cytokine levels 24 h after IL-2 application.*

cancer patients, not only Th1-like cytokines but also the production of Th2-like cytokines (especially IL-4) could be suppressed. Since most of our controls were below the detection limit, this hypothesis requires a more sensitive assay. The anti-inflammatory cytokine IL-10 was found at 12-fold higher levels in ascites than in the serum. This could indicate that tumours mainly exert their IL-10 immunosuppressive effect locally.

Concentrations of IL-2. Different IL-2 application routes resulted in different serum IL-2 concentrations per 1 MIU infused after 24 h. The highest values were found after administration in the peritoneal cavity, the second highest after intratumoural *(i.tu.)* injection and the lowest after injection in to the hepatic artery. However, our study did not clarify whether lower values represent slower leakage from tissue to the serum or faster clearance from the serum. Nevertheless, our data point to significant differences in the pharmacokinetics of IL-2 applied through different routes.

One day after IL-2 application, increased IL-2 levels were found both in the serum and ascites. Prior to therapy, ascitic levels of IL-2 were similar to those in the serum. Twenty-four hours after *i.p.* injection, the IL-2 levels had increased 534-fold in ascites, but in the same patients and time-points, they had increased only 10-fold in the serum. No differences were found between the first and the second injections. When all the data were considered, overall a 5-fold increase of IL-2 was found in the serum.

After treatment with IL-2, changes in serum and ascitic IL-2 levels are probably mainly due to the applied IL-2. In contrast, the levels of other cytokines are probably induced by IL-2.

Pharmacokinetic studies had revealed that, after *i.p.* application, IL-2 was slowly transported to the blood and high concentrations of IL-2 could be achieved for 24 h both in the peritoneal fluid and in the serum (26). These data were confirmed by our results, although the precise pharmacokinetics were difficult to determine in our study since different amounts of IL-2 were injected, close to or in different tumour tissue. However, our results indicated that, after 24 h, most of the IL-2 remained where it had been injected; local accumulation is, of course, the major difference between local and systemic IL-2 therapy. High local concentrations of IL-2 could explain the dramatic vascular leakage effect resulting in massive tumour cell death at the injection site (27). Since local vascular leakage appears to be related to the clinical efficiency of local IL-2 therapy (27), local accumulation of IL-2 may be crucial for both the therapeutic effect and lack of side-effects during local IL-2 therapy.

No changes in IL-4 and TNF-α levels. The cytokines IL-4 and TNF-α were not increased by local IL-2 therapy. Even on analysing each individual patient, no marked increases in IL-4 and TNF-α were found. TNF-α has been indicated as an effector mechanism in systemic IL-2 therapy (28). TNF-α was also suggested to be a putative secondary effector of local IL-2 therapy. (29-32) However, TNF-α was not produced in our patients.

Minimal induction of IL-10. In cancer patients, the IL-10 levels showed 1.4- and 1.7-fold increases in serum and a 1.9-fold increase in ascites after IL-2 applications, respectively. The IL-10 concentrations were increased in orders of magnitude after systemic administration of IL-2 (33-35) and in some trials after prolonged regional administration (36). Our local IL-2 applications induced only a marginal increase in IL-10. This minor increase of the immunosuppressive IL-10 could explain the therapeutic difference between local and systemic IL-2 therapy since IL-10 levels rise strongly after systemic IL-2 therapy. IL-10 indirectly inhibited the production of Th1-like cytokines through inhibitory effects on macrophages/monocytes (15, 16, 37) and suppressed the production of IFNγ and TNFα. IL-10 is an important immunosuppressive factor in cancer. (38-40). Thus, the relative absence of IL-10 induction by local IL-2 therapy when compared to systemic IL-2 therapy could be a factor contributing to favourable therapeutic outcomes.

Late effects on IFNγ. As stated above, serum IFNγ was significantly decreased in the patients prior to IL-2 therapy, compared to the healthy controls. Even 24 h after IL-2 injections, this was still the case. However, one month after the start of IL-2 therapy, the serum IFNγ levels were similar to those in the healthy controls. IFNγ is considered to be an important effector cytokine in anticancer therapy, which correlates with tumour regression (41, 42) and cellular anticancer effectors (43). Restoration of the cellular immune response could be important for the general condition of patients, but the observed response was probably not strong enough for therapeutic effects.

Local IL-2 therapy induces and boosts IL-5 levels. Both IL-2 injections induced a significant serum increase in IL-5 levels; the increase after the second injection (9-fold) was significantly higher than after the first injection (3-fold) ($p=0.02$), indicating that repeated IL-2 injections potentiate IL-5 production. One day after IL-2 application, the ascitic IL-5 levels were 9-fold higher compared to the serum. Thus, the strongest effects of IL-5 were local. All these data together point to an important role of IL-5 downstream of local IL-2.

In other studies, IL-5 was increased concomitantly with eosinophils (26, 44, 45) and eosinophils are attracted by IL-5 (46, 47). Eosinophils were found after systemic IL-2 therapy (45) and locally after successful local IL-2 therapy (4). IL-5 can be produced by type 2 cytotoxic T lymphocytes, which cooperate with IFNγ of type 1 cytotoxic T lymphocytes in tumour rejection (48, 49). Significant cytotoxicity against tumour cells can also be expressed by eosinophils after indirect IL-5-mediated *in vivo* activation by IL-2 (50).

Conclusion

The production of different cytokines can be suppressed in tumour patients. This applies to IL-2, IL-4, IL-5, IFNγ and TNFα. IL-2 applications at the site of tumours induced

increase in the local and systemic levels of IL-2, IL-5 and IL-10. IL-2 and IL-5 increased strongly in the serum and even more locally in ascites (at the site of the abdominal tumours), while IL-10 increased mildly and mainly locally. This suggests that these cytokines enter the serum through some leakage, but that the injected cytokines and their effects remain mainly local. By acting on different cells, IL-2 and IL-5 may influence the antitumour response in clinical trials. The increased levels of IL-5 in all patients, which were even higher locally at the injection site, indicated that IL-5 may be a downstream mediator of antitumour responses during local IL-2 therapy. The relatively minor increase of the immunosuppressive IL-10 could be therapeutically important.

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